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Characterization of the Haloacid Dehalogenase from *Xanthobacter autotrophicus* GJ10 and Sequencing of the *dhlB* Gene

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The haloacid dehalogenase of the 1,2-dichloroethane-utilizing bacterium *Xanthobacter autotrophicus* GJ10 was purified from a mutant with an eightfold increase in expression of the enzyme. The mutant was obtained by selecting for enhanced resistance to monobromoacetate. The enzyme was purified through $(NH_4)_2SO_4$ fractionation, DEAE-cellulose chromatography, and hydroxylapatite chromatography. The molecular mass of the protein was 28 kDa as determined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and 36 kDa as determined with gel filtration on Superose 12 fast protein liquid chromatography. The enzyme was active with 2-halogenated carboxylic acids and converted only the L-isomer of 2-chloropropionic acid with inversion of configuration to produce D-lactate. The activity of the enzyme was not readily influenced by thiol reagents. The gene encoding the haloacid dehalogenase (*dhlB*) was cloned and could be allocated to a 6.5-kb *Eco*RI-*Bgl*II fragment. Part of this fragment was sequenced, and the *dhlB* open reading frame was identified by comparison with the N-terminal amino acid sequence of the protein. The gene was found to encode a protein of 27,433 Da that showed considerable homology (60.5 and 61.0% similarity) with the two other haloacid dehalogenases sequenced to date but not with the haloalkane dehalogenase from *X. autotrophicus* GJ10.

Hydrolytic dehalogenases are key enzymes in the detoxification of aliphatic halogenated hydrocarbons. They catalyze the cleavage of carbon-halogen bonds through a nucleophilic substitution by water to yield an alcohol. At least two distinct groups can be recognized with respect to their substrate ranges: haloalkane dehalogenases hydrolyze halogenated alkanes, whereas haloacid dehalogenases are active with short-chain 2-halogenated carboxylic acids.

Of the 2-haloacid dehalogenases (E.C. 3.8.1.2), a number of enzymes have been purified and characterized (14, 16, 17, 19, 22, 29, 34). They have been divided in different classes according to their substrate specificity (9), electrophoretic mobility on polyacrylamide gels (9, 36), and stereospecific action on 2-monochloropropionic acid (2-MCPA) (36). Four different types of dehalogenation of 2-MCPA can be recognized. Two of these are represented by enzymes that are active with only L- or D-2-MCPA, giving products with inverted configuration at the chiral carbon atom. The other two act on both isomers, one with inversion of configuration and the other with retention of configuration.

Previous studies have shown the presence of more than one haloacid dehalogenase in the same organism (9, 16), with only minor differences in substrate specificities. It has been suggested that these isoenzymes have arisen by gene duplication and subsequent divergent evolution (9).

We are interested in the relation between structure, enzymatic mechanism, and evolution of dehalogenases. To study these aspects, we started to investigate the haloacid dehalogenase of *Xanthobacter autotrophicus* GJ10. This bacterium was isolated on 1,2-dichloroethane as the sole carbon and energy source (13). The organism is able to grow on a variety of halogenated aliphatic compounds and possesses, in addition to a haloacid dehalogenase, a haloalkane dehalogenase. This raises the question of whether haloacid dehalogenases and haloalkane dehalogenases are related by evolution or mechanistically related.

In a previous paper, we reported the cloning of both dehalogenase genes from GJ10 (12). The haloalkane dehalogenase has been purified (15) and crystallized (25), and its sequence (12) and tertiary structure (5) have been determined. The haloacid dehalogenase gene has been cloned on a 10-kb fragment in the broad-host-range cosmid vector pLAFR1 (12). Here, we describe the purification and characterization of the haloacid dehalogenase from an overproducing mutant of GJ10 and the sequence of the gene encoding this enzyme. The properties of the enzyme are compared with those of other haloacid dehalogenases and haloalkane dehalogenase.

MATERIALS AND METHODS

Growth conditions. Strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown in LB medium (20) at 30 or 37°C. *Xanthobacter* strains were grown at 30°C in MMY medium ([13] with 10 mg of yeast extract per liter replacing the vitamins) containing 5 mM carbon source in closed flasks. Stock solutions (0.5 or 1 M) of haloalkanoic acids were neutralized and sterilized by passage through 0.45-μm-pore-size filters. For plates, 1.5% agar was added. The antibiotics used for selection of strains were tetracycline (12.5 μg/ml), ampicillin (50 μg/ml), and kanamycin (50 μg/ml).

For purification of the enzyme, strains were grown at 60% oxygen saturation (air atmosphere) in a 10-liter fermentor (Braun Biostat E; Melsungen, Germany) in MMY medium at 30°C with 1.0% citrate as the carbon source and supplemented with yeast extract (100 mg/liter) and tetracycline (12.5 mg/liter). The pH was kept at 7.0 with 50% (wt/vol) citric acid. After 2 days of growth, extra $(NH_4)_2SO_4$ (15 g) and yeast extract (1 g) were added.

Isolation of a dehalogenase-overproducing mutant. Mutants of *X. autotrophicus* GJ10 resistant to monobromoacetate

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant features	Reference or source
X. autotrophicus		
GJ10	Wild type, DhlB$^+$	12
XD	Wild type, DhlB$^-$	11
GJ10M50	Haloacid dehalogenase-overproducing strain	This study
E. coli		
HB101	RecA$^-$, used for transformation with pLAFR1 derivatives	2
JM101	Δ(*lac-proAB*) *lacI*^{q} *lacZM15*; used for transformation with pLAFR3 and pGEM derivatives	39
Plasmids		
pLAFR1	Tc^r Tra$^-$ Mob$^+$, RK2 replicon	6
pLAFR3	Derivative of pLAFR1 containing multiple cloning site of pUC7 *dhlB*, pLAFR1	31
pPJ66	*dhlB*, pLAFR1	12
pPJ67	8.3-kb *Bgl*II fragment of pPJ66 in *Bam*HI site of pLAFR3	This study
pPJ69	6.5-kb *Eco*RI-*Bgl*II fragment of pPJ66 in pLAFR3	This study
pPJ94	7.3-kb *Eco*RI fragment of pPJ66 in pLAFR3	This study
pPJ95	3.4-kb *Pst*I deletion in pPJ94	This study
pPJ96	3.5-kb *Kpn*I deletion in pPJ94	This study
pPJ97	2.9-kb *Pst*I fragment of pPJ94 in pLAFR3	This study
pGEM7Zf(−)	Sequencing vector	Promega
pPS1	6.5 kb *Bgl*II-*Eco*RI fragment of pPJ66 cloned in pGEM7Zf(−)	This study
pPS5	*Apa*I deletion subclone of pPS1	This study
pPS7	5.3-kb *Eco*RV fragment of pPJ66 cloned in pGEM7Zf(−)	This study
pRK2013	*tra*(RK2), ColE1 replicon, Km^r	4

(MBA) were isolated by plating out cells of GJ10 on MMY plates containing 5 mM citrate and 10 mM MBA. Colonies that appeared were restreaked and purified on plates with the same composition.

Preparation of crude extracts and enzyme purification. Crude extracts from 0.2- to 1-liter cultures were prepared from cells grown to the late-exponential phase as described by Janssen et al. (11).

For the purification of haloacid dehalogenase, cells from the 10-liter culture were harvested when the optical density at 450 nm had reached 23. The cells (40 g [dry weight]) were collected by centrifugation (11,000 × *g* for 15 min) after 5 days of growth, washed, and suspended in 1,500 ml of 10 mM Tris-SO_4 (pH 7.5) containing 1 mM EDTA and 1 mM β-mercaptoethanol (TEM buffer). All further operations were carried out at 4°C in buffers that contained 1 mM EDTA and 1 mM β-mercaptoethanol to protect the enzyme against inactivation.

Cells were disrupted by sonication for 10 min (100 ml in an Ultrasonics W-375 sonicator at 300 W output) under permanent cooling. A crude extract was obtained after unbroken cells and debris had been removed by centrifugation for 20 min at 24,000 × *g*.

The cell extract (2.9 mg/ml) was fractionated with $(NH_4)_2SO_4$ in two steps. $(NH_4)_2SO_4$ was added to 35% saturation, and the precipitate was removed by centrifugation. The supernatant was brought to 60% $(NH_4)_2SO_4$ saturation, and after 15 min the precipitate was collected and dissolved in TEM buffer. The enzyme solution was dialyzed against the same buffer for 22 h.

The dialyzed enzyme solution (420 ml) was applied to a DEAE-cellulose column (2 by 27 cm) that had been equilibrated with TEM buffer. The column was washed with TEM buffer, and elution was carried out with a gradient of 0 to 0.5 M $(NH_4)_2SO_4$ in TEM buffer (total volume, 600 ml; fraction volume, 10 ml; flow rate, 35 ml/h). Active fractions eluted at 0.02 to 0.2 M $(NH_4)_2SO_4$ and were pooled and dialyzed against 5 mM potassium phosphate buffer (pH 6.5) for 18 h.

A precipitate formed in the dialysate was removed by centrifugation (15,000 × *g* for 15 min) before the dialyzed enzyme was applied to a hydroxylapatite column (1.5 by 18 cm) equilibrated with 5 mM potassium phosphate (pH 6.5). The column was washed with 20 ml of the same buffer, and the enzyme was eluted with a linear gradient of 5 to 200 mM potassium phosphate (pH 6.5). Active fractions eluted without retention and were pooled.

The enzyme solution was applied to a DEAE-cellulose column (1 by 18 cm) equilibrated with TEM buffer. The column was washed with 250 ml of 10 mM Tris-SO_4 (pH 7.5), and elution was carried out with a linear gradient of 0 to 0.1 M $(NH_4)_2SO_4$ in TEM buffer (total volume, 400 ml; flow rate, 30 ml/h; fraction volume, 5 ml). The active fractions eluted from 0.03 to 0.05 M ammonium sulfate and were concentrated to 6 mg/ml with an ultrafiltration membrane (Diaflo PM30; Amicon).

Enzymatic assays. Haloacid dehalogenase activities were routinely measured with monochloroacetate as the substrate. A suitable amount of the enzyme solution was incubated in 3 ml of 5 mM substrate in 50 mM glycine-NaOH (pH 9.0) at 30°C. Liberation of halide was followed spectrophotometrically as described by Bergmann and Sanik (1). One unit of dehalogenase activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of halide per min.

D- and L-lactate were determined by monitoring NADH production with D- and L-lactate dehydrogenase, respectively, as described by Neilands (24).

Protein was measured with Coomassie brilliant blue with bovine serum albumin as a standard.

Estimation of molecular mass. The molecular mass of the denatured protein was estimated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (15).

Superose-12 (Pharmacia) gel filtration high-performance liquid chromatography was used to estimate the molecular mass of the native protein. The column was equilibrated with TEM buffer at 21°C. Proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. The column was calibrated with bovine serum albumin (66,000), ovalbumin (43,000), trypsin inhibitor (20,100), and cytochrome *c* (12,500) as molecular weight standards.

N-terminal sequence analysis. For determination of the N-terminal amino acid sequence, partially purified enzyme from strain XD(pPJ66) was used. An SDS–10% polyacrylamide gel containing 0.1 mM thioglycolic acid with a stacking gel consisting of 2% agarose was preelectrophoresed at 40 mA for 6 h. An amount of 0.75 mg of partially purified enzyme was loaded. After electrophoresis, the proteins were electroblotted onto a polyvinylidene difluoride membrane (21) and briefly stained with Coomassie brilliant blue. Destaining was with 45% methanol–10% acetic acid–45% H_2O (vol/vol). The protein band containing dehalogenase was cut

from the membrane, and the N-terminal part of the protein was determined by gas-phase sequencing (Applied Biosystems model 477A sequencer) by Eurosequence, Groningen, the Netherlands.

DNA manipulations and genetic procedures. Standard DNA techniques (20) were used for plasmid isolation, restriction enzyme digestion, ligation, and transformation. Mobilization of pLAFR1 derivatives to *Xanthobacter* sp. XD was by the triparental mating procedure described previously (12) with pRK2013 as a helper plasmid (4).

DNA sequencing and analysis. Unidirectional deletions in plasmid DNA were generated by exonuclease III digestion with the procedure of Henikoff (10). Double-stranded DNA from overlapping plasmids was sequenced by the dideoxy-chain termination method of Sanger et al. (26) with [α-^{35}S]dATP (Amersham) with the universal and reverse pUC/M13 primers.

Analysis of nucleotide sequence obtained was with the PC/GENE program (Genofit, Geneva, Switzerland) and the Staden package (30). Protein sequences were compared with the SWISS-PROT protein data base (EMBL, Heidelberg, Germany) release 17 with the FASTA program (18). Protein sequences were aligned with the program BestFit from the University of Wisconsin Genetics Computer Group package (3).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank under accession no. M81691.

RESULTS

Isolation of a dehalogenase-overproducing mutant of GJ10. Mutants of *X. autotrophicus* GJ10 that were resistant to MBA were isolated by spreading cells on plates containing 5 mM citrate and 10 mM MBA. The wild-type strain is not able to grow with this concentration of MBA. One of the colonies that appeared after 3 to 4 weeks was purified on citrate plates with 10 mM MBA and designated strain GJ10M50. The mutant was able to grow on plates with 5 mM citrate and 35 mM MBA.

The haloacid dehalogenase activity in crude extracts of GJ10M50 grown in MMY with 5 mM citrate and 5 mM monochloroacetate was 4.8 U/mg of protein, compared with 0.6 U/mg in the wild-type strain. The relative activities with monochloroacetate, 2-MCPA, MBA, and dichloroacetic acid had not changed. This suggests that the mutant overexpresses the same dehalogenase as that produced by strain GJ10.

Purification of the haloacid dehalogenase. The haloacid dehalogenase was partially purified from *X. autotrophicus* XD harboring the haloacid dehalogenase-encoding plasmid pPJ66 (12). However, it was not possible to obtain pure protein in large amounts. Because the dehalogenase content of strain GJ10M50 is about three times higher than the dehalogenase level of XD(pPJ66), the former strain was used for purification of the enzyme.

TABLE 2. Purification of 2-haloacid dehalogenase

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg of protein)	Yield (%)	Purification factor
Crude extract	5,074	16,110	3.18	100	
Ammonium sulfate	1,741	11,892	7.26	74	2.3
DEAE-cellulose	711	7,012	9.86	44	3.1
Hydroxyapatite	91	4,744	52.1	29	16.4
DEAE-cellulose	47	2,833	60.3	18	18.9

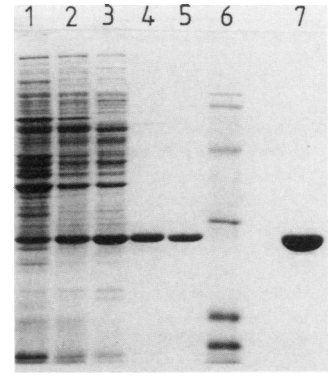

FIG. 1. SDS-PAGE of haloacid dehalogenase containing fractions during purification. Lanes: 1, crude extract of GJ10M50; 2, $(NH_4)_2SO_4$ fractionation; 3, first DEAE-cellulose fraction; 4, hydroxylapatite; 5, second DEAE-cellulose fraction (8 μg of protein applied); 6, marker proteins (Fig. 2); 7, 40 μg of purified protein.

The purification scheme for the haloacid dehalogenase from the mutant GJ10M50 is shown in Table 2. The enzyme was purified about 20-fold, which implies that the protein was present at about 5% of the total cell-free protein of the mutant. In the wild-type strain this will be 0.5 to 1%. When the preparation was applied to SDS-PAGE, a single protein band was visible (Fig. 1).

The dehalogenase activities of purified enzyme from strain GJ10M50 and of crude extract from strain XD(pPJ66) with different substrates are given in Table 3. The data show that the substrate specificities were the same. The enzymes also showed the same electrophoretic mobility after SDS-PAGE (Fig. 2) and after native PAGE (data not shown). These results indicate that the haloacid dehalogenases of strain GJ10M50 and XD(pPJ66) are identical. Unless stated otherwise, the dehalogenase purified from strain GJ10M50 was used in all further experiments.

Characterization of the enzyme. The molecular mass of the protein was determined to be 28 kDa with SDS-PAGE (Fig. 1). Fast protein liquid chromatography on a Superose-12 column indicated a molecular mass of 38 kDa.

The N-terminal sequence of the dehalogenase determined from the enzyme obtained from XD(pPJ66) was Met-Ile-Lys-

TABLE 3. Substrate specificity of the enzyme

Substrate	Activity (%)[a]	
	GJ10M50	XD(pPJ66)
Monochloroacetate	100	100
Dichloroacetate	134	131
Bromoacetate	98	100
Dibromoacetate	371	405
D,L-2-Chloropropionate	92	105
2,3-Dichloropropionate	5	
D,L-2-Chlorobutyrate	3	

[a] The rates of halide release are expressed as the percentage of the rate with monochloroacetate, which was 55.5 U/mg of protein for the pure enzyme of strain GJ10M50 and 1.5 U/mg of protein for crude extract of strain XD(pPJ66). With the pure enzyme, no activity was found with trichloroacetate, 2,2-dichloropropionate, 3-chloropropionate, 2-chloroacetamide, chloroacetone, or chloroacetaldehyde.

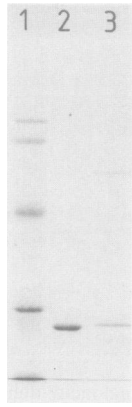

FIG. 2. Electrophoretic mobility of dehalogenases purified from strain XD(pPJ66) and strain GJ10M50 on SDS-PAGE. Lanes: 1, marker proteins (ovotransferrin [78 kDa], albumin [66 kDa], ovalbumin [45 kDa], carbonic anhydrase [30 kDa], and myoglobin [17.2 kDa]; 2, dehalogenase purified from GJ10M50; 3, dehalogenase purified from XD(pPJ66).

Ala-Val-Val-Phe-Asp-Ala-Tyr-Gly-Thr-Leu-Phe-Asp-Val-Gln-Ser.

The activity of the enzyme for various halogenated substrates was assayed by determination of the initial rates of halide release upon incubation with 5 mM substrate (Table 3). Only compounds with a halogen substituted at the α position were hydrolyzed. The highest activity was found with dibromoacetate. No activity was found for chloroacetamide, indicating the importance of the presence of a carboxyl group in the substrate.

After prolonged incubation of the enzyme with a racemic mixture of D,L-2-MCPA, half of the amount of chlorine present in the substrate was released as chloride (Table 4). After incubation with L-2-MCPA, equimolar amounts of D-lactate and chloride appeared in the solution; no L-lactate could be detected. Incubation under the same conditions with D-2-MCPA did not result in the release of chloride, and neither D-lactate nor L-lactate was detected. This shows that the enzyme is specific for L-2-MCPA and converts this compound with inversion of configuration at the chiral carbon atom.

Several possible enzyme inhibitors were tested (Table 5). The substrate analog trichloroacetic acid was not a strong inhibitor of enzyme activity. The enzyme was only slightly inhibited by thiol reagents.

The activity of the enzyme was measured at various pHs from 5 to 11 (Fig. 3). Maximum activity was found at pH 9.5.

Location and sequencing of the gene encoding the dehalogenase. Plasmid pPJ66 (12) was used for the generation of subclones to localize the region encoding the dehalogenase more precisely (Fig. 4). Because we were not able to detect dehalogenase expression from its own promoter in *E. coli* strains harboring pLAFR derivatives with the haloacid dehalogenase gene (*dhlB*), plasmids were conjugated to strain *X. autotrophicus* XD, which lacks a haloacid dehalogenase and is unable to grow with halogenated carboxylic acids. Transconjugants were tested for growth on monochloroacetate or 2-MCPA. The results indicated that the *dhlB* gene is located near one of the *Pst*I sites. A 6.5-kb *Bgl*II-*Eco*RI fragment was cloned in pLAFR3, and the resulting plasmid (pPJ69) was transferred to XD. Transconjugant XD(pPJ69) was able to grow with 2-MCPA.

The same *Bgl*II-*Eco*RI fragment was cloned in the *Bam*HI-*Eco*RI sites of the vector pGEM-7Zf(−). The resulting plasmid (designated pPS1) was digested with *Apa*I and ligated again to delete a 0.5-kb *Apa*I fragment containing an *Xho*I site (pPS5). Plasmid pPS5 was digested with *Sac*I to generate a 3′ protruding end and with *Xho*I to generate a 5′ protruding end. Unidirectional deletions from the *Xho*I site

TABLE 4. Stereospecificity of haloacid dehalogenase

Substrate	Chloride released (mM)	D-Lactate produced (mM)	L-Lactate produced (mM)
2 mM D,L-2-MCPA	0.95	0.99	ND[a]
1 mM D-2-MCPA	ND	ND	ND
1 mM L-2-MCPA	0.98	1.03	ND

[a] ND, not detectable.

TABLE 5. Effect of inhibitors on enzyme activity[a]

Inhibitor	Concn (mM)	Relative activity (%)
None		100
p-Chloromercuribenzoate	0.01	100
	0.1	79
N-Ethylmaleimide	0.1	103
	1	105
$HgCl_2$	0.01	69
	0.1	52
$ZnSO_4$	1	80
$MnSO_4$	1	100
EDTA	1	101
Trichloroacetate	10	56
	50	23

[a] Purified enzyme was incubated for 5 min at 30°C with the compound indicated. Residual activity was measured after the addition of 5 mM monochloroacetate and is expressed as the percentage of activity found without the inhibitor.

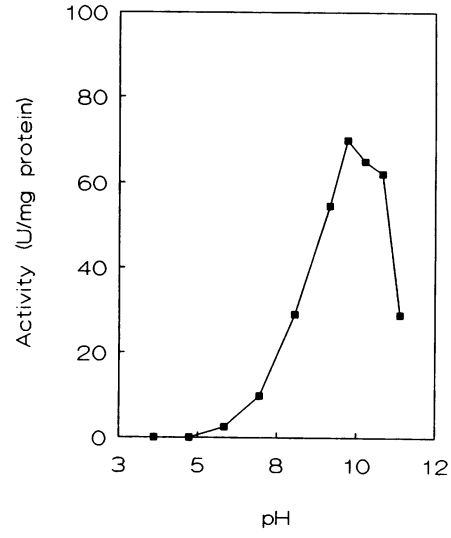

FIG. 3. Effect of pH on enzyme activity. Activity was determined at different pHs in the following buffers: sodium acetate (pH 4 and 5); potassium phosphate (pH 6 and 7); Tris-H_2SO_4 (pH 8 and 9); glycine-NaOH (pH 9.5, 10, 10.5, and 11).

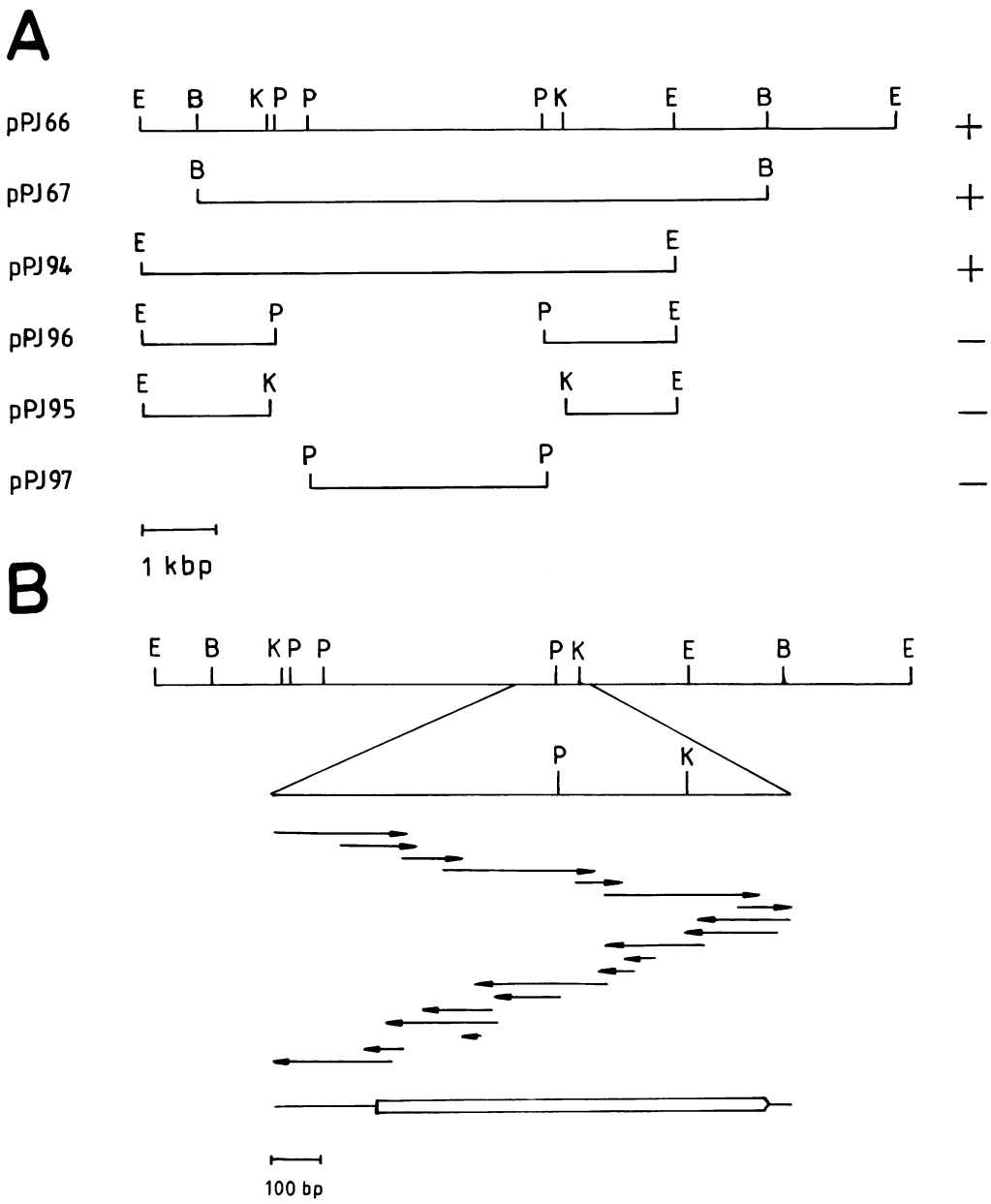

FIG. 4. Subcloning and sequencing of the *dhlB* region. (A) Subclones of pPJ66 and the ability of transconjugants of XD to grow with monochloroacetate. In all subclones, pLAFR1 or pLAFR3 was used as the vector. Restriction sites: E, *Eco*RI; B, *Bgl*II; P, *Pst*I; K, *Kpn*I. (B) Sequence strategy. The part of the plasmid that has been sequenced in both directions is shown enlarged. Arrows represent the direction and extent of sequencing. Only the region where both strands were sequenced is shown. The location and direction of the *dhlB* open reading frame are indicated by a bar.

in the insert were made with exonuclease III. After ligation and transformation, plasmids with inserts differing by approximately 300 bp in length were isolated and sequenced with the reverse pUC/M13 primer.

Analysis of the sequence information obtained indicated an open reading frame with a predicted N-terminal amino acid sequence that was completely identical to the N-terminal sequence obtained from the purified protein. The predicted molecular mass of the putative protein was 27,433 Da, which is very close to the value of 28 kDa obtained from SDS-PAGE.

To sequence the opposite strand, the 5.3-kb *Eco*RV fragment from pPJ66 was cloned into the *Sma*I site of pGEM-7Zf(−), and the resulting plasmid (pPS7) was digested with *Sac*I and *Hin*dIII. Progressive deletions from the *Hin*dIII site were generated with exonuclease III. In Fig. 4, the sequencing strategy of the *dhlB* region is shown.

The nucleotide sequence of the *dhlB* gene and the deduced amino acid sequence are shown in Fig. 5. No sequence upstream of *dhlB* that had similarity with the *E. coli* consensus promoter sequence of Mulligan et al. (23) could be found. A possible ribosome binding site close to the *dhlB* start codon was present.

The G+C content of the *dhlB* region was 67%. Compari-

```
                                                                    60
TTCCCAGTCGTGAATCCGGCCATTGCGAGCGTGCCTATTGGCTTCCTCGGCGCCATTCTC
                                                                   120
GGCGCACTGCTCTCGCCGCGGGATGCGGTTTCCGAGGCGCAGTTCGACGAGGTGGTCTTT
                                                                   180
CGCGCCAACACCGGGCTGCGCGATGATGCGCAGGCCGGCAAGAGCCTTCACTGATCAAGA
                                                                   240
TCCCGAGACAGCGAGAGGAACGACATGATCAAGGCAGTCGTGTTCGACGCTTACGGTACG
                xxxx      MetIleLysAlaValValPheAspAlaTyrGlyThr
                                                                   300
CTCTTCGACGTCCAGTCGGTGGCCGACGCCACCGAGCGGGCGTATCCAGGCCGGGGCGAG
LeuPheAspValGlnSerValAlaAspAlaThrGluArgAlaTyrProGlyArgGlyGlu
                                                                   360
TACATCACGCAGGTCTGGCGGCAGAAGCAGCTGGAATACAGCTGGCTCCGCGCGCTGATG
TyrIleThrGlnValTrpArgGlnLysGlnLeuGluTyrSerTrpLeuArgAlaLeuMet
                                                                   420
GGGCGCTATGCCGACTTTTGGGGCGTCACGCGGGAAGCGCTGGCCTATACCCTCGGAACG
GlyArgTyrAlaAspPheTrpGlyValThrArgGluAlaLeuAlaTyrThrLeuGlyThr
                                                                   480
CTGGGGCTGGAGCCGGACGAGTCCTTCCTCGCCGGGATGGCGCAGGCCTACAACCGCCTC
LeuGlyLeuGluProAspGluSerPheLeuAlaGlyMetAlaGlnAlaTyrAsnArgLeu
                                                                   540
ACGCCCTATCCGGACGCCGCGCAATGCCTCGCGGAGCTGGCGCCCCTCAAGCGCGCCATC
ThrProTyrProAspAlaAlaGlnCysLeuAlaGluLeuAlaProLeuLysArgAlaIle
                                                                   600
CTCTCCAACGGCGCGCCCCACATGCTGCAGGCGCTCGTGGCCAATGCGGGCCTGACGGAC
LeuSerAsnGlyAlaProHisMetLeuGlnAlaLeuValAlaAsnAlaGlyLeuThrAsp
                                                                   660
AGCTTCGATGCCGTCATCAGCGTCGATGCCAAGCGCGTGTTCAAGCCTCATCCCGACTCC
SerPheAspAlaValIleSerValAspAlaLysArgValPheLysProHisProAspSer
                                                                   720
TACGCGCTGGTGGAGGAGGTACTAGGCGTGACGCCCGCGGAGGTGCTGTTCGTGTCCTCC
TyrAlaLeuValGluGluValLeuGlyValThrProAlaGluValLeuPheValSerSer
                                                                   780
AACGGCTTCGACGTCGGCGGCGCGAAGAATTTCGGCTTCAGCGTCGCCCGGGTCGCGCGC
AsnGlyPheAspValGlyGlyAlaLysAsnPheGlyPheSerValAlaArgValAlaArg
                                                                   840
CTGTCGCAGGAGGCGCTGGCGCGCGAACTCGTCTCGGGTACCATCGCGCCCCTGACCATG
LeuSerGlnGluAlaLeuAlaArgGluLeuValSerGlyThrIleAlaProLeuThrMet
                                                                   900
TTCAAGGCGCTGAGGATGCGGGAAGAAACCTATGCGGAGGCGCCTGATTTCGTGGTGCCC
PheLysAlaLeuArgMetArgGluGluThrTyrAlaGluAlaProAspPheValValPro
                                                                   960
GCCCTTGGCGACCTGCCGCGGCTGGTTCGCGGGATGGCCGGCGCTCATCTCGCACCAGCG
AlaLeuGlyAspLeuProArgLeuValArgGlyMetAlaGlyAlaHisLeuAlaProAla

GTGTGACGAGGCATGGCCCCGATGGAGGAGATGGGCGGCGGCCATTGGCATGACC
Val***
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FIG. 5. Nucleotide sequence of the *dhlB* region and the deduced amino acid sequence of the haloacid dehalogenase. Symbols: xxxx, putative ribosome binding site; ***, stop codon.

son of the *dhlB* amino acid sequence with the SWISS-PROT data bank yielded no proteins with significant homology. However, there was homology with the recently published sequences of two haloacid dehalogenase genes of *Pseudomonas* sp. strain CBS3 (27). The amino acid sequence of the *dhlB*-encoded protein was 61.0 and 60.5% similar to the products of *dehCI* and *dehCII* from this strain, respectively. The alignment of the three protein sequences is given in Fig. 6.

DISCUSSION

We purified the 2-haloacid dehalogenase from strain *X. autotrophicus* GJ10M50, which has about eightfold-higher activity than the wild-type strain. The mutant was isolated on citrate plates containing 10 mM MBA, a concentration which is toxic for the wild type, and could tolerate a concentration of 35 mM MBA. Probably, the higher dehalogenase activity lowers the concentration of MBA in the cell, thereby reducing its toxic effects. Weightman et al. (37) also isolated mutants with dehalogenase activities higher than those of the wild-type strain, but these mutants showed increased sensitivity to halogenated alkanoic acids. Moreover, mutants selected by Weightman et al. (37) for increased resistance to MBA were found to have a decreased dehalogenase activity. These researchers suggested the association of dehalogenase genes with permease genes; mutations occurring in one permease gene could have polar effects (28). Strotmann et al. (32) isolated a mutant of a 2-chloroethanol-utilizing *Pseudomonas* strain that was resistant to high concentrations of 2-chloroethanol. This mutant had completely lost haloacid dehalogenase activity, and it was thought that an exporting permease reduced high concentrations of intracellular haloalkanoic acids. Thus, in different organisms resistance to haloalkanoic acids may be correlated with either increased or decreased dehalogenase content. At present, we are investigating MBA-resistant mutants and the possible function of a permease involved in haloalkanoic acid catabolism in more detail.

A number of haloacid dehalogenases have been purified to date. They may be divided according to their substrate range, stereospecific action on D,L-2-MCPA, stereochemical configuration of the product, susceptibility to SH reagents,

```
                    1
GJ10 dhlB      MIKAVVFDAYGTLFDVQSVADATERAYPGRG    31
CBS3 dehCI   MDPIRACVFDAYGTLLDVNTAVMKHAHDIGGCA
CBS3 dehCII  MQEIRGVVFDLYGTLCDVHSVAQLCGQYFPERG
                *.  ***.**** **  ..        .
          2
EYITQVWRQKQLEYSWLRALMGRYADFWGVTREALAYTLGTLGLEP    77
EELSSLWRQRQLEYSWTRTLMGRYADFWQLTTEALDFALESFGLLE
TEISLMWRQKQLEYSWLRSLMGQYVSFPQATEDALVFVCNALNLKL
. .. .***.******.*.*** *..*  .* .** .. ....*
                                    3
DESFLAGMAQAYNRLTPYPDAAQCLAEL--APLKRAILSNGAPHML   121
RTDLKNRLLDAYHELSAYPDAVGTLGALKAAGFTTAILSNGNNEML
REDTRIALCNEYLNIKPYREVRSALESLRSGAVPLAILSNGSAHSI
 ..    . ..*  ...*...   *..*  .    ******.   .

QALVANAGLTDSFDAVISVDAKRVFKPHPDSYALVEEVLGVTPAEV   166
RGALRAGNLTEALDQCISVDEIKIYKPDPRVYQFACDRLDVRPSEV
QSVVGNAGIEHFFSHLISADEVSVSKPSPAAYELAEKRLKVVRSKL
. ..    ... ..  **.*. .. ** *  *... . * * ....
         4
LFVSSNGFDVGGAKNFGFSVARVARLSQEALARELVSGTIAPLTMF   212
CFVSSNAWDIGGAGAFGFNTVRINRIN-------------------
LFVSSNAWDASGARHFGFQVCWVNRSRNT-----------------
 *****..* .**  *** . ...* .

KALRMREETYAEAPD-FVVPALGDLPRLVRGMAGAHLAPAV        253
-----KPQEYSFAPQRHQLSSLSELPQLLLRLTQ
------FEQLGERPD-HVISGLDELPNLLNFASADR
            *.   ...*..** *.
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FIG. 6. Amino acid sequence comparison of L-2-haloacid dehalogenases from *X. autotrophicus* GJ10 (*dhlB*) and *Pseudomonas* sp. strain CBS3 (*dehCI* and *dehCII*). Symbols: *, identical residue; . , similar residue; –, gap introduced for horizontal alignment. The upper lines indicate the four highly conserved regions.

and electrophoretic mobility under nondenaturing conditions. The haloacid dehalogenase from GJ10 was found to convert only the L-isomer of 2-MCPA with inversion of configuration and was relatively insensitive to thiol reagents. These properties are also exhibited by the enzymes described by Goldman et al. (7), Klages et al. (16), Little and Williams (19), and Tsang et al. (34). The enzyme described by Smith et al. (29) has essentially the same properties, apart from its stereospecificity. This enzyme was active only with D-2-MCPA. It is of interest to note that up to now strains that contain only an L-2-haloacid dehalogenase (e.g., GJ10) or that contain both an L-2-haloacid dehalogenase and a D-2-haloacid dehalogenase have been described, but to our knowledge no strain that possesses only a D-2-haloacid dehalogenase has yet been isolated.

The haloacid dehalogenase of strain GJ10 has some similarities with the recently described haloalcohol dehalogenase from *Arthrobacter* sp. strain AD2 (35). The molecular mass of this enzyme was 29 kDa in its denatured state, and its amino acid composition was about the same as that of the haloacid dehalogenase. The haloalcohol dehalogenase was not active with haloacids, but chloroacetic acid and chloropropionic acids acted as inhibitors of enzyme activity. Antibodies raised against the enzyme did not react with the haloacid dehalogenase. There was no homology between the N-terminal amino acid sequences of the two proteins. Whether the similarity is fortuitous or is the result of an evolutionary relationship or structural similarity remains to be established.

The gene encoding the enzyme could be located by introduction of plasmids in *Xanthobacter* sp. strain XD, which lacks dehalogenase activity (11). Unlike the case with the haloalkane dehalogenase gene (*dhlA*) of *X. autotrophicus* GJ10, we could not detect any expression of *dhlB* from its own promoter in *E. coli* strains. It is not clear whether this is due to the absence of a promoter sequence that can be recognized by RNA polymerase of *E. coli* or whether some additional genetic elements are neccessary for expression. Greer et al. (8) observed a growth phase dependence of dehalogenase expression in *X. autotrophicus* GJ10, which indicates the presence of some regulatory elements. It may well be that plasmid pPJ66 possesses, apart from *dhlB*, other genes whose products are necessary for growth on haloalkanoates, for example, a permease or transport protein or a regulatory protein. In agreement with this, some induction of dehalogenase synthesis by dichloroacetic acid was observed in strain XD(pPJ66) (12). We are currently analyzing the region upstream of the haloacid dehalogenase gene for the presence of genes involved in substrate transport or regulation of halocarboxylic acid catabolism.

The amino acid sequence encoded by *dhlB* showed considerable homology with the sequences of the two *Pseudomonas* sp. strain CBS3 haloacid dehalogenases, which are both active only with L-2-MCPA. The properties of the

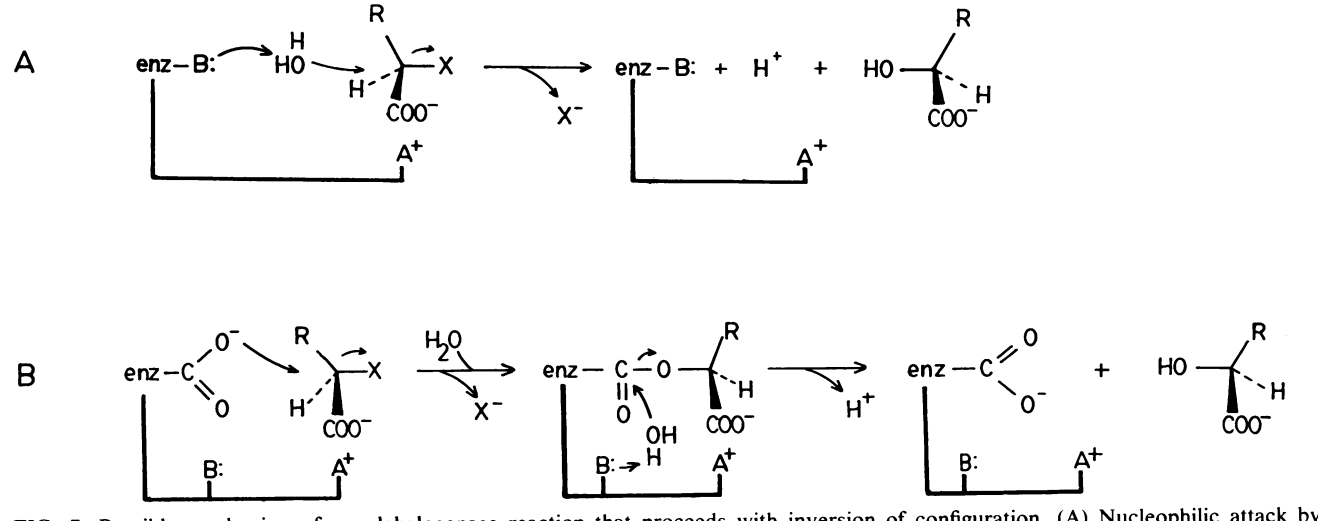

FIG. 7. Possible mechanisms for a dehalogenase reaction that proceeds with inversion of configuration. (A) Nucleophilic attack by activated water; (B) attack by a carboxylate group of the enzyme followed by ester hydrolysis.

enzyme encoded by *dehCI* (16) are indeed very similar to those of strain GJ10. The electrophoretic mobilities of the dehalogenases from CBS3 and from strain GJ10 were different, however (34), indicating that this method does not classify rather similar dehalogenases in the same group. We propose that there is a distinct class of L-2-MCPA-specific dehalogenases that convert their substrate with inversion of configuration and that these enzymes are related through evolution and mechanistically similar.

Two different mechanisms for hydrolytic dehalogenation that is accompanied by inversion may be envisaged (7, 19, 36). First, water could be activated and directly carry out a nucleophilic displacement of the halogen (Fig. 7A). The second mechanism proposes that a carboxylate group from aspartate or glutamate acts as the nucleophile, leading to the formation of an ester intermediate that is then hydrolyzed by an attack of water on the carbonyl carbon (Fig. 7B). The three-dimensional structure of the haloalkane dehalogenase of strain GJ10 supports the latter mechanism (5). For both mechanisms, one would expect to find in the sequence at conserved positions one or more positively charged amino acids for binding the carboxylate group of the substrate (36) and also an amino acid that can accept a proton from water around the pH optimum of the enzyme. The second mechanism would, in addition, require a conserved glutamate or aspartate. Comparison of the three dehalogenase sequences (Fig. 6) shows that there are four conserved regions. Several positively charged amino acids, amino acids that could accept a proton, and acidic amino acids are present at conserved positions. Conserved histidines and cysteines are absent, however. From the sequence comparison, it is thus not possible to rule out either of the mechanisms shown in Fig. 7.

There was no sequence homology of *dhlB* with *dhlA*, the gene encoding haloalkane dehalogenase from the same organism. It is interesting that the G+C content of *dhlB* is 67%, about the same as the G+C content of the *X. autotrophicus* chromosome (38), whereas the G+C content of *dhlA* is only 58%. This might indicate that, in *X. autotrophicus*, the haloacid dehalogenase has evolved earlier than the haloalkane dehalogenase. It has recently been found that the haloalkane dehalogenase gene is plasmid encoded, whereas the haloacid dehalogenase gene is on the chromosome of strain GJ10 (33).

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